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Membrane Topology and Mutational Analysis of the TolQ Protein of *Escherichia coli* Required for the Uptake of Macromolecules and Cell Envelope Integrity

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TolQ is a 230-amino-acid protein required to maintain the integrity of the bacterial envelope and to facilitate the import of both filamentous bacteriophage and group A colicins. Cellular fractionation experiments showed TolQ to be localized to the cytoplasmic membrane. Bacteria expressing a series of TolQ–β-galactosidase and TolQ-alkaline phosphatase fusion proteins were analyzed for the appropriate enzyme activity, membrane location, and sensitivity to exogenously added protease. The results are consistent with TolQ being an integral cytoplasmic membrane protein with three membrane-spanning regions. The amino-terminal 19 residues as well as a small loop in the 155 to 170 residue region appear exposed in the periplasm, while the carboxy terminus and a large loop after the first transmembrane region are cytoplasmic. Amino-terminal sequence analysis of TolQ purified from the membrane revealed the presence of the initiating formyl methionine group, suggesting a rapid translocation of the amino-terminal region across the cytoplasmic membrane. Analysis of various *tolQ* mutant strains suggests that the third transmembrane region as well as parts of the large cytoplasmic loop are necessary for activity.

The outer membrane of *Escherichia coli* acts as a permeability barrier against antibiotics, bile salts, and digestive enzymes. Only small hydrophilic molecules (<600 Da) are able to cross the outer membrane by simple or facilitated diffusion (30). However, there are systems which facilitate the uptake of larger molecules. For example, ferric siderophores, vitamin B_{12}, group B colicins, and DNA of some phages (φ80, T1) are translocated across the outer membrane with the help of the TonB and ExbBD proteins (32). TonB and ExbD are located in the periplasm and anchored to the cytoplasmic membrane via an N-terminal hydrophobic sequence (6, 10, 33). ExbB is also an inner membrane protein with three membrane-spanning segments, but most of it is exposed to the cytoplasm (11, 14). ExbB appears to interact with TonB and stabilize it from proteolytic digestion (34) during its synthesis or export (14). A second interaction between ExbBD and TonB occurs in the inner membrane and involves the N-terminal membrane-spanning part of TonB (13).

The TolQRAB proteins are components of another system which appears to be involved in the maintenance of cell envelope structure. Mutations in any of the *tolQRAB* genes render the cell sensitive to drugs like bile salts and induce the release of periplasmic enzymes (16–18). In addition, the TolQRA proteins are required for infection by filamentous bacteriophages and sensitivity to group A colicins, while TolB is only involved in the entry of some group A colicins like colicin A (35, 40). Although the Tol system facilitates the uptake of these bacteriocins, no normal transport function has been assigned to the Tol proteins.

The Ton and Tol proteins share many common features. The sequences of TolQR and ExbBD are highly homologous in their membrane-spanning regions (7). Cross-complementation occurs between these proteins to stabilize TonB (4, 5). We have recently shown that the topology of TolR is similar to that of ExbD (29). The TolA and TonB proteins consist of an N-terminal membrane-spanning fragment, the remaining part of the proteins being periplasmic (6, 21, 23, 33). Like TonB, TolA is thought to entirely span the periplasm to interact with some components of the outer membrane (23). The C-terminal domain of TolA appears to be the region involved in such interactions (23) and is connected with the N-terminal membrane anchor via a long α-helical region (21). Although TolA and TonB do not show global sequence similarity, the N-terminal parts of both molecules are functionally homologous, since the TolA N-terminal sequence is able to promote efficient TonB translocation (13).

In this report, we present the topology of TolQ and the characterization of *tolQ* mutations altered in the cytoplasmic region and in the third membrane-spanning domain of TolQ.

MATERIALS AND METHODS

Growth media, strains, and plasmids. The following *E. coli* K-12 strains were used in this study: 1292 (*supE hsdS met gal lacY tonA*, from A. Wood), JC5039 (1292 *tolQ856*), JC5041 (1292 *tolQ925*), JC5042 (1292 *tolQ890*), GM1 (P90c/F'*lac pro*), TPS13 [GM1 *tolQ13*(Am)], TPS66 (GM1 *tolQ66*), CC118 [*araD* Δ(*ara-leu*)*7697* Δ*lacX94 phoA* Δ*20 galE thi rpsE rpoB argE*(Am) *recA-1* (27)], CC170 (CC118 carrying a Tn*lacZ* chromosomal insertion [25]), and K38 HfrC, transformed with plasmid pGP1-2, K17(DE3)plysS (22). Plasmid pJC185 con-

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tained the *ORF1tolQRAB'* *Eco*RI-*Bam*HI 4,319-bp region (22, 35). To construct plasmid pJC402, the 1,849- to 3,748-bp *Hin*dIII-*Bst*EI fragment of pJC185 was replaced by a 1.5-kb *Hin*dIII-*Bst*EI fragment of pPHO7 (8) containing part of the *phoA* gene allowing in vitro generation of fusion. Plasmids pT7-5 and pGP1-2 (carrying the T7 RNA polymerase under the control of the left promoter of lambda) have been described elsewhere (36). The *Eco*RI-*Hpa*I, *Hin*cII-*Nco*I, and *Eco*RI-*Hin*dIII fragments of pJC185 were subcloned into pT7-5 to give plasmids pT7-1QRA, pT7-Q, and pT7-1Q. These plasmids were able to express the *ORF1*, *tolQ*, *tolR*, or *tolA* genes under the control of the T7 promoter. Plasmid pTLQ1, which consists of the *Eco*RI-*Nco*I fragment from the pTPS304 (35) subcloned into pET3 (21), encodes *tolQ* under control of the T7 promoter. For dominance-complementation analysis, *Eco*RI-*Hin*dIII fragments containing *ORF1*, *tolQ* mutations, and *tolR* were subcloned into pJEL126 (38). The copy number of this plasmid is one per genome at low temperatures (30 to 37°C). Cells were grown in LB medium (28). Ampicillin (100 μg/ml) and kanamycin (50 μg/ml) were added to the medium to maintain the plasmids.

Enzyme assays. Enzymatic assays for alkaline phosphatase (37) and β-galactosidase (28) activities have been described previously. The periplasmic location of the alkaline phosphatase moiety of fusion proteins was also detected by the characterization of blue colonies on plates containing 40 μg of 5-bromo-4-chloro-3-indolyl phosphate per ml, purchased from Research Organics, Inc.

Recombinant DNA techniques. Purification of DNA, treatment with restriction or modification enzymes, and agarose gel electrophoresis were performed as described by Maniatis et al. (24). DNA fragments were recovered from agarose by using the Geneclean kit (Bio 101, La Jolla, Calif.).

Tn*phoA* and Tn*lacZ* in vivo mutagenesis. Tn*phoA* and Tn*lacZ* in vivo mutagenesis procedures were performed by the methods of Manoil and Beckwith (27) and Manoil (25), respectively. Cells transformed with pJC185 or pTLQ1 were mutagenized, and clones were selected for growth on ampicillin and high-concentration kanamycin (300 μg/ml) to isolate plasmids carrying a transposon element. Each Tn*phoA* or Tn*lacZ* insertion was characterized by sequencing the junction region between genes *tolQ* and *phoA* or *lacZ*.

Linker insertion mutagenesis. pT7-1QRA was partially digested with *Cfo*I. The linker AGCTCG was ligated to the purified linear plasmid by the method of Barani (1, 2).

In vitro generation of *tolQ-phoA* fusions. Plasmid pJC402 was double digested with *Kpn*I and *Hin*dIII enzymes. Unidirectional deletions were generated by digesting the open plasmid with exonuclease III at room temperature by using the nested-deletion kit (Pharmacia, Upsala, Sweden) as recommended by the manufacturer.

T7 expression, compartmentation, and protease accessibility of TolQ. pT7-5 derivatives carrying the *tol* genes were used to express the corresponding Tol proteins from K38 cells containing pGP1-2 after heat induction of the T7 RNA polymerase and labeling of the cells by 10 μCi of [^{35}S]methionine, in the presence of rifampin by the method of Tabor (36). For conversion into spheroplasts, cells were resuspended in 50 μl of 10 mM Tris-HCl (pH 8.1)–20% sucrose–5 mM EDTA–lysozyme (0.02 mg/ml). After incubation for 30 min on ice, $MgSO_4$ was added at a final concentration of 16 mM to stabilize the spheroplasts. After centrifugation (10 min at 10,000 × *g*), the supernatant was taken as the periplasmic fraction. The pellet was washed with 100 μl of 10 mM Tris-HCl (pH 8.1)–20% sucrose–10 mM $MgSO_4$. Lysis was achieved by suspension of the spheroplasts in 50 μl of 10 mM Tris-HCl (pH 8.1)–5 mM EDTA and by rapid freezing and thawing of the cells. DNase I was added to a final concentration of 0.04 mg/ml. The membrane fraction was pelleted by centrifugation (30 min at 13,000 × *g*). The supernatant was taken as the cytoplasmic fraction, and the membrane fraction was washed with 10 mM Tris, pH 8.1.

For protease accessibility experiments, cells were spheroplasted as above but they were not centrifuged; the cytoplasm was made accessible after solubilization of the membranes by addition of 1% Triton X-100. One portion of each fraction was treated with proteinase K (0.1 mg/ml). After incubation for 45 min on ice, the reaction was stopped by the addition of 3 mM phenylmethylsulfonyl fluoride.

For pulse-chase experiments, cells were labeled for 5 min, and nonradioactive methionine (50 mM) was added and samples were withdrawn after 0, 30, 60, and 180 min of the chase period. Proteins were separated on a sodium dodecyl sulfate (SDS)–15% polyacrylamide gel (15) and detected after fluorography. The peptide sizes were determined with rainbow markers (Amersham, Amersham, England).

Protease accessibility of TolQ-PhoA fusions. Protease digestion of TolQ-PhoA fusions at TolQ residues 70 and 155 was performed in the following manner. A 100-ml culture of K17(DE3)plysS containing plasmids encoding TolQ-PhoA fusions was grown at 37°C to 2 × 10^8 cells/ml. The fusion protein was induced with 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 10 min. Cells were harvested by centrifugation, and spheroplasts were prepared as described previously (9). The spheroplasted cells were split into two 5-ml samples: one was treated with 10 μl of chymotrypsin (5 mg/ml). After incubation for 30 min on ice, the reaction was stopped by the addition of lima bean trypsin inhibitor to a final concentration of 50 μg/ml. The spheroplasts were lysed, and the membranes were separated, as described by Guy-Caffey et al. (9). The cytoplasmic membrane fraction was pulled from the isopycnic sucrose density gradient and isolated by centrifugation (Beckman TY 65 rotor at 45,000 rpm for 3 h). The membrane pellet was solubilized overnight in 0.25 M Tris (pH 6.8)–4% SDS. The protein concentration of each membrane sample was determined by the bicinchonic acid assay (Pierce Chemical Co.). Equal amounts of total protein were run on an SDS-10% polyacrylamide gel (15). The fusion protein was detected by Western blot (immunoblot) with antibody to alkaline phosphatase and ^{125}I-protein A.

Purification of TolQ, and amino-terminal sequencing. A 770-bp fragment extending from the *Eco*RI site upstream of *tolQ* to the codon for TolQ amino acid 228 and followed by an engineered *Xho*I site was generated from pTPS304 (35) by PCR. This fragment was ligated into pET21(+) from Novagen, Inc., to generate pTLQHis. This plasmid allows IPTG-inducible expression from the T7 promoter of TolQ with a C-terminal 6XHis tag. Purification of TolQHis was carried out in the following manner. K17(DE3) containing pTLQHis was grown to 2 × 10^8 cells/ml and induced with 2 mM IPTG for 60 min. The cells were pelleted and resuspended in a 1/16 volume 1× binding buffer (20 mM Tris [pH 7.9]–500 mM NaCl–5 mM imidazole) and then lysed by two passages through a French pressure cell at 1,200 lb/in^2. Membranes were pelleted onto a sucrose cushion as described previously (9). The membrane fraction was pulled from the sucrose cushion and solubilized overnight in a final volume of 80 ml of 1× binding buffer—6 M guanidine hydrochloride (GuHCl). Insoluble material was removed by centrifugation (Beckman 50.2 Ti rotor at 18,000 rpm for 20 min), and the solubilized membranes were then incubated overnight with 15 ml of His · Bind resin (Novagen, Inc.) which had been charged with nickel sulfate as recom-

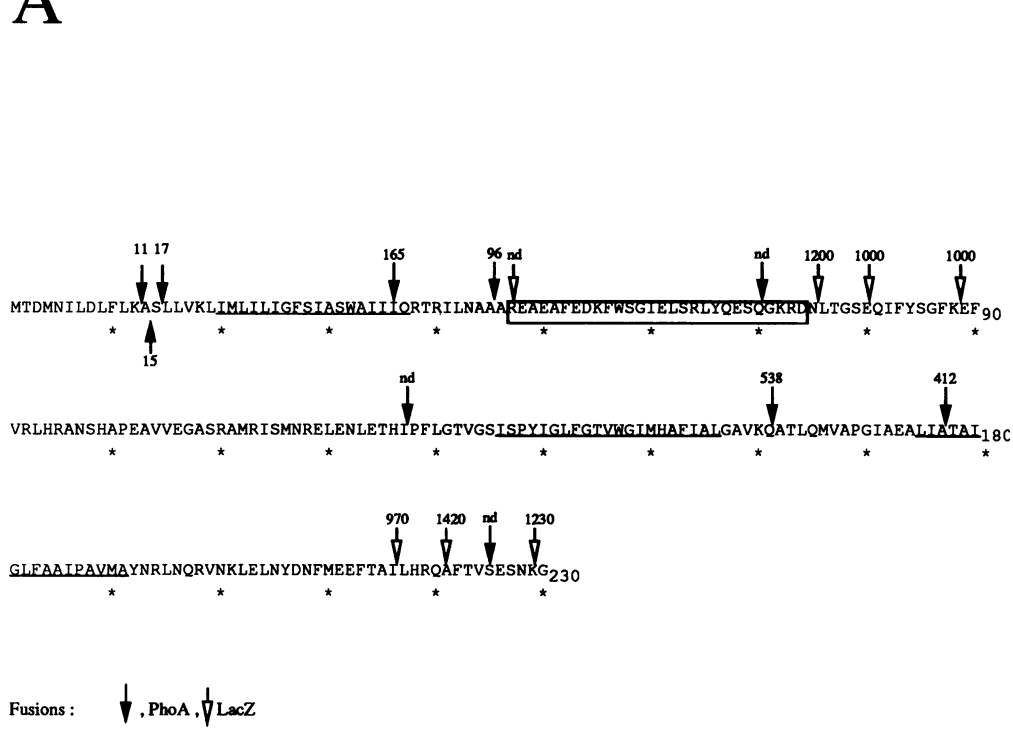

FIG. 1. (A) Sequence of the TolQ protein. The membrane-spanning regions are underlined and the cytoplasmic charged region is boxed. The positions (arrows) of the different fusions as well as their activities (numbers) are indicated. nd, not determined. (B) Proposed transmembrane arrangement of TolQ (230 amino acids) in the inner membrane. The N-terminal end is located in the periplasm, and the C-terminal part of TolQ is cytoplasmic. The positions of the *tolQ* mutations are indicated (arrows). The membrane-spanning segments are indicated by heavy lines.

mended by the manufacturer. The column was poured at 0.5 ml/min, washed first with 45 ml of 1× binding buffer–6 M GuHCl, and then washed again with 45 ml of 1× binding buffer–50 mM imidazole–6 M GuHCl. TolQHis was eluted with a 50 to 400 mM imidazole gradient, and 2-ml fractions were collected. TolQHis eluted at a 240 to 300 mM imidazole concentration and could be detected by silver staining of a 10 to 15% SDS-Phast Gel (Pharmacia). Fractions containing TolQHis were concentrated, and then the protein was precipitated out of the 6 M GuHCl solution by the addition of 40 volumes of absolute ethanol followed by incubation at −20°C for 1 h. The protein was pelleted by centrifugation (Sorvall SS-34 rotor at 10,000 rpm for 45 min) and then subjected to preparative SDS-polyacrylamide gel electrophoresis (PAGE) (10 to 16%). Left, right, and central strips of the gel were removed and Coomassie blue stained. The unstained gel strips were aligned, the 25-kDa band corresponding to TolQHis was excised, and the protein was isolated by electroelution.

For amino-terminal sequencing, 25 μg of gel-purified TolQHis was subjected to SDS–12% PAGE and then electroblotted to Hyperbond in 10 mM mixture of CAPS [3-(cyclohexylamino)-1-propane-sulfonic acid] (Sigma, C-2632), pH 11, and 10% methanol. The Hyperbond was stained with 0.1% Coomassie brilliant blue R-250 for 10 min. Destaining was

done in 25% ethanol–10% acetic acid until bands could be visualized clearly. The band corresponding to TolQHis was excised carefully and divided into three equal samples. The first sample was placed in 100% methanol for 2 h at room temperature, the second was placed in 0.5 N HCl in methanol for 2 h at room temperature, and the last sample was treated with 3 N HCl for 2 h at 37°C. The acid-treated samples were then neutralized with 1 M $NaHCO_3$, pH 9.5. All the samples were washed thoroughly with water before amino-terminal sequence analysis.

The N-terminal sequence of the electroblotted samples was determined with a Porton 2090 gas phase sequencer interfaced to a Beckman System Gold high-performance liquid chromatograph for the on-line detection of the PTH amino acids. Each excised membrane was cut to fit the reaction cartridge, overlayed with a protein support disk and a Zitex membrane, and subjected to 13 cycles of automated Edman degradation chemistry by standard procedure 1 supplied with the sequencer. PTH amino acids were detected at 269 nm. The overall repetitive yields (estimated to be 93 to 96%) and picomoles of initial sequenceable material were determined from the slope and intercept (respectively) of the linear regression analysis of a plot of the log picomoles of PTH amino acid versus the cycle number.

RESULTS

Membrane topology of TolQ. The sequence of *tolQ* predicts that the TolQ protein contains three membrane-spanning fragments (35). We analyzed the membrane topology of TolQ by isolating *tolQ-phoA* and *tolQ-lacZ* fusions (26). Alkaline phosphatase fusions are active only when the hybrid protein is exposed to the periplasm, while fusions with β-galactosidase are active in the cytoplasm. *tolQ-phoA* fusions with various amounts of alkaline phosphatase activity could be isolated in regions of positions 12 to 45 and 155 to 177 of TolQ, while fusions with no alkaline phosphatase activity were isolated in the region of positions 69 to 128 and at the C-terminal part of the protein (Fig. 1A). Highly active *tolQ-lacZ* fusions were isolated in these last two regions, confirming their cytoplasmic localization. TolQ-PhoA fusions at residues 12 to 14 had rather low activity levels, suggesting that these proteins probably did not insert into the membrane. Practically all of the data are consistent with the proposed transmembrane arrangement of TolQ shown in Fig. 1B, with the amino-terminal end located in the periplasm and the carboxyl-terminal part in the cytoplasm. The exception appears to be with the TolQ-PhoA fusions at residues 36 to 45 which produced substantial alkaline phosphatase activity. This suggests that the first predicted hydrophobic stretch of TolQ (residues 20 to 38) can act as an efficient signal or export sequence when another protein is fused to it. Consistent with this result, the TolQ-LacZ fusion with Arg47 did not show any activity; this probably reflected a membrane location of this fusion.

The TolQ-PhoA hybrid proteins were recovered mainly in the inner membrane fraction after cell fractionation except for the fusions at residues 12 to 14, which remained in the cytoplasm. For example, fusion proteins containing PhoA fused to amino acids 70 and 155 of TolQ were present in isolated cytoplasmic membranes (Fig. 2, lanes 1 and 3), even though only the TolQ-PhoA fusion protein at residue 155 shows alkaline phosphatase activity in intact bacteria. To further substantiate the location of PhoA in these fusions, the protease accessibility of the PhoA moiety in these fusions was determined in spheroplasts. Bacterial cultures containing each of these fusion proteins were subjected to digestion with chymotrypsin, and the cytoplasmic membranes were isolated (9). Equal amounts of total protein from each membrane preparation were analyzed for the presence of the PhoA moiety by Western blot analysis using anti-PhoA antibody. Chymotrypsin was used, since the sequence of the linker region (from the IS*50L* of Tn*5*) which precedes the PhoA enzymatic portion of each fusion is susceptible to cleavage with this protease. Only the PhoA moiety of the TolQ-PhoA fusion at residue 155 was cleaved in spheroplasts, indicating its periplasmic location (Fig. 2, compare lanes 2 and 4). These data suggest that the short loop formed by residues 157 to 174 is located in the periplasm (Fig. 1).

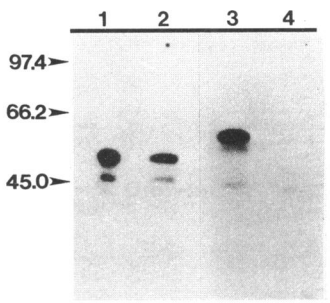

FIG. 2. Chymotrypsin accessibility of TolQ-PhoA fusions. Cells containing plasmids for TolQ-PhoA fusions at TolQ residues 70 (lanes 1 and 2) and 155 (lanes 3 and 4) were induced with IPTG for 10 min and then converted to spheroplasts. After incubation in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of chymotrypsin, cytoplasmic membranes were isolated and subjected to SDS-PAGE and Western blot analysis using antibody to alkaline phosphatase. The predicted molecular sizes for fusion proteins TolQ69-PhoA and TolQ155-PhoA are 53.6 and 63.1 kDa, respectively. Protein standards in kilodaltons are marked (arrowheads).

The membrane location of hybrid proteins containing PhoA fused to residue 70 of TolQ, together with the cytoplasmic location of the PhoA portion of this hybrid protein, is consistent with the hypothesis that the hydrophobic residues 19 to 37 span the cytoplasmic membrane as shown in Fig. 1B. This would place the amino-terminal 18 amino acids in the periplasm, as predicted by Bourdineaud et al. (3), provided there is no processing reaction cleaving this region. To determine the fate of the amino-terminal portion of TolQ, the protein containing a carboxyl-terminal polyhistidine tag (TolQHis) was purified from bacteria containing pTLQHis. This protein is found almost exclusively in the cytoplasmic membrane, even when overproduced (Fig. 3, left panel, lane 6). The purified protein, which migrated as one band on polyacrylamide gels (Fig. 3, right panel, lane 2), was subjected to N-terminal amino acid sequencing (Table 1). No terminal amino acids were detected unless the protein was treated with 0.5 N HCl in methanol or 3 N HCl, conditions which remove amino-terminal formyl groups (31, 41). Following acid treatment, the sequence obtained was that predicted from the DNA sequence and included the initiating methionine. These data suggest that the amino terminus is rapidly translocated across the membrane following synthesis with the unprocessed amino terminus residing in the periplasm.

To further analyze the topology of TolQ, protease accessibility experiments were done with K38 containing pT7-1Q and pGP1-2, which expresses the wild-type protein. TolQ was expressed and labeled with [^{35}S]methionine in the presence of rifampin. Spheroplasts and cell lysates from these bacteria were incubated with various concentrations of proteinase K and trypsin, and the resulting labeled TolQ fragments were

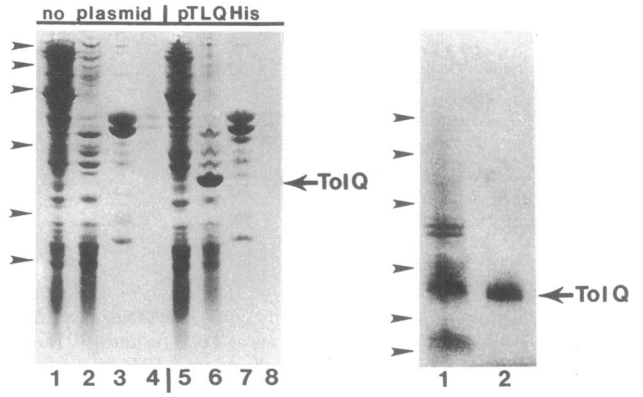

FIG. 3. Localization and purification of TolQHis. (Left panel) K17(DE3) or K17(DE3)/pTLQHis was treated with IPTG and the cells fractionated into inner and outer membranes as described by Guy-Caffey et al. (9). The proteins were separated by electrophoresis in the presence of SDS on a 12% polyacrylamide gel and stained with Coomassie blue. Each fraction contained the material obtained from the same number of bacteria. Lanes 1 to 4, fractions from K17(DE3) bacteria; lanes 5 to 8 from K17(DE3)/pTLQHis bacteria. Lanes 1 and 5, cytoplasmic and periplasmic material; lanes 2 and 6, cytoplasmic membranes; lanes 3 and 7, outer membranes; lanes 4 and 8, dense material containing inclusion bodies. The arrowheads on the left mark the positions (from the top) of protein standards of 97.5, 66.2, 45, 31, 21.5, and 14 kDa. (Right panel) Analysis of the products on SDS gradient-polyacrylamide gradient gels (10 to 15%) from guanidine extraction of cytoplasmic membranes from induced K17(DE3)/pTLQHis bacteria (lane 1) and purified TolQHis (lane 2). The arrows indicate the positions of the same protein standards shown in the left panel.

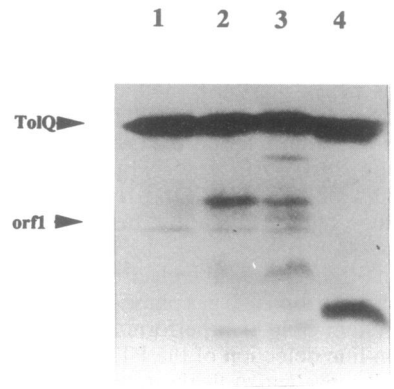

FIG. 4. Accessibility of TolQ to proteinase K and trypsin in spheroplasts and cell lysates. Cells were labeled and converted to spheroplasts or cell lysates. Addition of proteinase K or trypsin to the cells at a concentration of 0.1 mg/ml was followed by incubation for 30 min on ice. Lane 1, spheroplasts without protease; lane 2, without proteinase K; lane 3, without trypsin; lane 4, cell lysates with proteinase K. Rainbow markers were used to determine the molecular weight of each polypeptide.

analyzed by SDS-PAGE (Fig. 4). TolQ (25.5 kDa) was degraded to 23.5-, 17-, 13-, 11.5-, and 5.5-kDa products after digestion with proteinase K. Five fragments of 21, 17, 16, 13, and 7.5 kDa were obtained after digestion with trypsin. Most of the partially digested bands were degraded when the spheroplasts were lysed (Fig. 4, lane 4). The Orf1 protein was located in the cytoplasm because it was only digested in cell lysates. The 23.5- and 21-kDa products indicated that the N terminus was accessible to both proteases, while the 17-, 16-, and 5.5-kDa fragments are consistent with the presence of the periplasmic residue 155 to 170 region. The 13- and 11.5-kDa fragments are probably secondary digestion products of the 17-kDa fragment. Under our conditions, not all the TolQ molecules were cleaved, since a portion of the 25.5-kDa peptide remained intact even at higher proteinase K concentrations (1 mg/ml). This could be the result of incorrect folding or integration of some TolQ molecules through overexpression of the T7 promoter in the presence of rifampin. Nevertheless, the interpretation of the digestion data is consistent with the model with the fusions elaborated in Fig. 1.

Characterization of *tolQ* mutations. Previously described chromosomal mutations were cloned by using a DNA probe corresponding to the wild-type *tolQ* gene and sequenced (19, 35). All the *tolQ* mutants were altered in cell envelope integrity (Table 2). However, there were differences in the sensitivity of the various *tolQ* alleles to phage and colicins. Cells carrying the *tolQ890* mutation corresponding to an alteration in the *tolQ* ribosome binding site or the *tolQ925* mutation were fully sensitive to phage f1 and colicin E1 but resistant to colicin A. Cells carrying a *tolQ856* mutation were only partially resistant to colicins and phages.

The mutant TolQ proteins were labeled with [^{35}S]methionine in the presence of rifampin (36) after induction at high temperature (see Materials and Methods). Approximately equivalent amounts of TolQ, TolQ856, and TolQ925 proteins were present in the bacteria (Fig. 5). However, the TolQ856 and TolQ925 proteins migrated more rapidly than the wild-type TolQ protein. As expected, the *tolQ890* mutant synthesized a smaller amount of wild-type TolQ protein, since the mutation is located in the ribosome binding region.

tolQ mutants were isolated on a multicopy plasmid carrying the *orf1-tolQ-tolR-tolA* genes by TAB linker mutagenesis, which allows insertion of two amino acids at various positions of the TolQ protein (1, 2). We obtained insertions of the amino acids Arg-Ala at positions 46 (*tolQ522*), 92 (*tolQ514*), and 198 (*tolQ528*) of TolQ and an insertion of two serines at position 100 (*tolQ531*) (Fig. 1B). Complementation-dominance analyses between some of the *tolQ* alleles were carried out after

TABLE 1. Amino-terminal sequence analysis of TolQ

Treatment	N-terminal sequence	Sequenceable amt (~pmol)
100% methanol	No sequence detected[a]	0
0.5 N HCl in methanol	M-T-D-M-N-I-L-D-L	0.99
3 N HCl	M-X-D-M-N-I-L-D-L-F-L-K-A[b]	2.17
None (predicted)[c]	M-T-D-M-N-I-L-D-L-F-L-K-A-S.....	

[a] The control sample (no HCl pretreatment) gave no detectable sequence.
[b] The absence of threonine in the second cycle is probably due to the HCl treatment at 37°C.
[c] Amino-terminal sequence predicted for TolQ from DNA.

TABLE 2. Phenotype of *tolQ* chromosomal mutations

Mutation (position)	Sensitivity to cholic acid[a]	Alkaline phosphatase release (%)[b]	Relative sensitivity to:		
			Phage f1[c]	Colicin A[d]	Colicin E1[d]
Wild type	R	1.5	10^9	1	1
tolQ13 (stop after codon 36)	S	80.0	R	250	250
tolQ66 (G181D)	S	84.0	R	1,000	1,000
tolQ856 (G101R)	S	65.0	10^4	100	100
tolQ925 (A177V)	S	84.0	10^9	1,000	1
tolQ890 (RBS)[e]	S	55.0	10^9	100	1

[a] Ability (R) or inability (S) of the bacteria to grow on plates containing 2.5% cholic acid.
[b] Percent total alkaline phosphatase activity recovered in the growth medium. Less than 5% total β-galactosidase activity was recovered in the external medium under the conditions used.
[c] A stock of f1 phages was titered on each strain; the phage stock titer is given as particles per milliliter; R, no phage plaque could be seen at the highest phage concentration.
[d] Relative colicin sensitivity is expressed as the fold increase in concentration of colicin needed to give the same clear spot on plates as the wild type.
[e] RBS, ribosome binding site.

subcloning of the mutant alleles into pJEL126. Cells carrying *tolQ* mutations were transformed with such plasmids and grown at 30°C in the presence of ampicillin to allow the complementation-dominance analysis. *tolQ514* and *tolQ531* mutations complemented the *tolQ13*(Am) mutation, while *tolQ522*, *tolQ528*, *tolQ856*, *tolQ890*, and *tolQ925* did not (Table 3). Thus, small insertions in position 92 or 100 of TolQ did not affect the protein function in such conditions. However, the *tolQ514* mutation was unable to complement the *tolQ856*, *tolQ925*, and *tolQ890* mutations and the *tolQ531* mutation did not complement the *tolQ856* mutation. The *tolQ856* allele was not complemented by any of the other *tolQ* mutations tested except that the phenotype of colicin A tolerance due to the *tolQ925* mutation appeared to be dominant in such conditions. The *tolQ890* mutation was only complemented by the *tolQ514* and *tolQ531* alleles. This mutation was not complemented by a plasmid carrying the *ORF1tolQ* region but was complemented by a plasmid carrying *ORF1tolQR*. Thus, the alteration of the ribosome binding site of *tolQ* had a polar effect on the expression of *tolR*. The *tolQ66* mutation was also complemented by the *tolQ514* and *tolQ531* alleles. However, the phenotype of tolerance to colicins A and E1 exhibited by this mutation appeared to be dominant. An analysis of protein stability by pulse-chase experiments did not reveal any difference in the stability of all the altered proteins compared with TolQ.

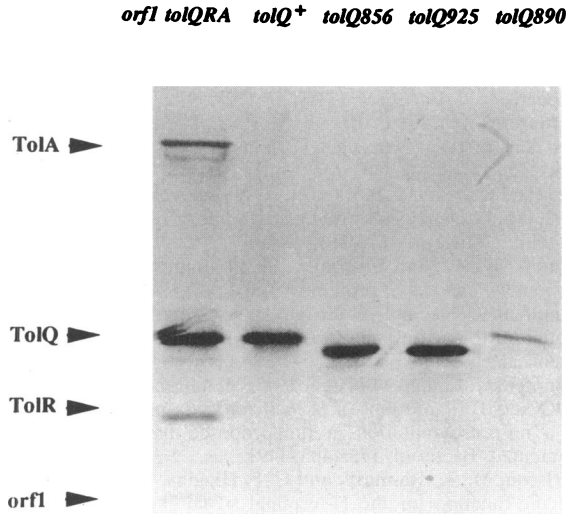

FIG. 5. Autoradiograph of [^{35}S]methionine-labeled TolQ-mutated proteins. Proteins were separated on an SDS–15% polyacrylamide gel and exposed overnight at −70°C after fluorography.

DISCUSSION

The data presented in this paper are consistent with TolQ being an integral cytoplasmic membrane protein with three membrane-spanning regions. The N terminus of the protein as well as a small loop in the residue 155 to 170 region of the protein are located in the periplasm, while the C terminus and a large loop after the first membrane-spanning region are cytoplasmic. The membrane topology of TolQ, determined by protease accessibility experiments, has been reported recently (12). Although the overall topology of TolQ is the same, our model assumes that the three membrane-spanning segments are located at residues 20 to 38, 136 to 156, and 175 to 193, while Kampfenkel and Braun (12) proposed that these segments are located at residues 9 to 36, 127 to 159, and 162 to 191. The main difference between these two models is the extent of the N-terminal periplasmic part. Our model places the two lysine residues at positions 12 and 18 in the periplasm on the basis of the trypsin accessibility experiment. Furthermore, this places fewer charged residues in the first transmembrane segment. This conformation is similar to that of ExbB, whose amino acid sequence is highly homologous to TolQ in its membrane-spanning region. Thus, the membrane topology of TolQ, TolR, and TolA is similar to that found for ExbB, ExbD, and TonB (10, 11, 32, 33).

The amino-terminal sequence analysis of TolQ isolated from membranes showed that almost all of the protein had retained its initiating formyl methionine group. This suggests that there is no processing of the amino-terminal end and that the first transmembrane region is rapidly inserted into the membrane. The resulting topology of the first transmembrane region is analogous to that of the same region of leader peptidase of *E. coli* (39, 42). Perhaps, the first transmembrane domain to TolQ is inserted into the membrane in a Sec-independent region, as is the case for the first transmembrane domain of leader peptidase (20).

Mutations located in the two cytoplasmic portions and third membrane-spanning region of TolQ have been characterized. The missense *tolQ66* mutation and the plasmid-encoded *tolQ522* and *tolQ528* mutations essentially exhibited a null phenotype similar to the *tolQ13*(Am) mutation; the phenotype of tolerance to colicins due to the *tolQ66* mutations was

TABLE 3. Complementation-dominance analysis of *tolQ* mutations

pJEL126 derivative	Chromosomal mutation									
	tolQ13		*tolQ66*		*tolQ856*		*tolQ925*		*tolQ890*	
	Envelope integrity[a]	Sensitivity to colA, colE1[b]	Envelope integrity	Sensitivity to colA, colE1	Envelope integrity	Sensitivity to colA, colE1	Envelope integrity	Sensitivity to colA	Envelope integrity	Sensitivity to colA
pJEL126	−	−	−	−	−	+	−	−	−	+/−
tolQ$^+$	+	+	+	−	+	+	+	+	+	+
tolQ514	+	+	+	−	−	+	−	−	−	+
tolQ522	−	−	−	−	−	+	−	−	−	+
tolQ528	−	−	−	−	−	+	−	−	−	+
tolQ531	+	+	+	−	−	+	+	+	+	+
tolQ856	−	+	−	−	−	+	−	−	−	+
tolQ925	−	−	−	−	−	−	−	−	−	+
tolQ890	−	+	+	−	−	+	−	−	+	+

[a] Integrity of the cell envelope was controlled by testing the resistance or sensitivity to cholic acid and the release of periplasmic RNase and alkaline phosphatase; +, wild-type phenotype; −, sensitivity to cholic acid and release of periplasmic enzymes.
[b] The sensitivities of the mutants to colicins were assayed as described in the Table 2 footnotes: +, sensitive; −, resistant. colA, colicin A; colE1, colicin E1.

dominant. Other mutations, such as *tolQ856* and *tolQ925*, affected the uptake of colicin and filamentous phage to different extents. These results suggest that it may be possible to separate the import function of TolQ from its normal function in maintaining cell envelope integrity. Analysis of the *tolQ890* mutation, which is altered in the ribosome binding site of TolQ and produces lower amounts of the native proteins, clearly showed that this small amount was sufficient for phage and colicin import, while more TolQ protein is necessary to maintain the envelope integrity. This mutation had a polar effect on the expression of *tolR*. The separation of the functions of TolQ can result from the presence of specific functional domains within TolQ, or alternatively they may reflect an alteration of interaction with other proteins, like TolRAB, which are necessary for such functions.

The topology of the TolQRA proteins is very similar to that of the corresponding proteins in the Ton system. It has been reported that ExbB interacts directly with TonB (13). The observation that a fusion peptide composed of the membrane-spanning portion of TolA attached to the periplasmic portion of TonB appears to function more efficiently in the presence of TolQ (13) suggests that there may be a similar direct interaction between TolQ and TolA. Some interactions between TolQ and TolR or TolA likely involve the third membrane-spanning region of TolQ, as two TolQ mutations (*tolQ66* and *tolQ925*) located on the same face of this proposed helix effectively disrupt the function of the Tol system. It also has been shown that the cytoplasmic part of ExbB is necessary for the translocation and stability of TonB (13). It should be pointed out that these experiments were done in the presence of excess ExbB. However, in the *tolQ13*(Am) strain TPS13, addition of approximately normal amounts of wild-type TolQ had little if any effect on the amount of TolA present (data not shown). This would indicate that normal levels of TolQ in the absence of TolR have little effect on the stability of TolA. Further experiments are necessary to determine the exact interactions which occur between the Tol proteins during the import of macromolecules and maintenance of cell envelope integrity.

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